EQUILIBRIUM AND METASTABLE STATES IN LECITHIN FILMS

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ABSTRACT We have considered whether lecithin surface films below the gel-liquid crystal transition temperature, T_c, are in unique physical states. In general, below T_c, equilibrium films do not exist when surface pressures, π, exceed about 0.1 dyn/cm. Since surface pressure-surface area isotherms of lecithin films below T_c always encompass π's much greater than 0.1 dyn/cm, the film states are metastable. We show that the film properties under these conditions depend strongly on the history of the film, particularly the method of film formation. Lecithin surface films below T_c are thus in arbitrary metastable states, so that π-area isotherms are difficult to interpret. The physical significance of such isotherms remains to be determined. The utility of pure lecithin surface layers below T_c as models for biological systems is also challenged by our results.

INTRODUCTION

Monolayers containing lecithin have been used as models for studying phase relations in lipid bilayers and cell membranes (4, 5, 7, 11, 16, 17, 21), and for lung surfactant in the study of the mechanics of the lung (1, 6, 8, 12, 13, 20, 23). The significance of many of the studies with synthetic lecithins is, for us, uncertain because of the variability of the surface pressure (π) surface area (A) isotherms observed by different investigators (3, 7, 8, 17, 21–23). Obvious discrepancies among π-A isotherms determined in the same laboratory with different manufacture lots of the same material might reasonably be attributed to trace contaminants (17, 23), but unequivocal identification of specific contaminants or their mole fractions in the materials has proved very difficult. Thus it is not clear which data reported by other laboratories should be dismissed because of trace contamination. Differences in rates of film compression and other details of experimental protocol might also account for discrepancies but there is no rigorous way to analyze these factors at this time. However, we believe that the principal reason for the variability is that surface films often are not equilibrium structures, but can exist in any number of metastable states depending on the history of the surface film.

For equilibrium, surface films must conform to the following criteria: (*a*) values of π should never exceed that for a saturated solution of the lipid, i.e., the equilibrium spreading pressure, π_e; (*b*) the π-A isotherm can be generated reversibly; (*c*) the isotherm should be independent of the pathway of formation of the monolayer; and

(*d*) the system should obey Gibbs' phase rule. It is recognized that in many cases lecithin surface films do not satisfy these basic criteria (17, 18, 22). Rather it is thought that the films exist in metastable equilibrium and that the apparent phase transitions observed are transitions between two unique metastable states. It is of course necessary that the states of the model system be determined uniquely by prescribed intensive thermodynamic variables if we are to deduce principles from which useful insights to the biological system may be developed.

In this study the uniqueness of the surface film states of L-α-dipalmitoyl (DPL) and L-α-dimyristoyl (DML) lecithin is assessed with respect to some equilibrium and metastability criteria. In particular, we demonstrate that when films are formed at temperatures below the gel-liquid crystal transition temperature of lecithin, T_c, non-equilibrium states occur that are history-dependent. But at temperatures that exceed T_c, stable, reproducible surface films are formed that are independent of the pathway of film formation. The implications of these findings on molecular interpretations of phase transitions in lecithin films is discussed.

Two different methods of monolayer formation were used to check for pathway independence: (*a*) films spread from solvent at the temperature of measurement of the π-A isotherm, and (*b*) films spread from the liquid crystal phase and cooled to the same temperature. Temperatures were chosen to encompass T_c for DML (23.5°C). Studies with DPL were restricted to temperatures below T_c (41°C) because this T_c exceeds the upper temperature limit of the film balance used under which precise π-A data can be obtained.

MATERIALS AND METHODS

Chemicals were reagent grade and used without further purification. Water was prepared fresh daily by double-distillation in a quartz still. Chloroform was used as solvent for the lecithins. DPL and DML were obtained from Applied Science Labs, Inc. (State College, Pa.), and tested for purity. The fatty acids were 99.9% homogeneous by gas-liquid chromatographic analysis. Lysolecithin and other contaminants were not detected by thin layer chromatography of 200-μg samples.

Surface balance was the horizontal float type. The trough (60 × 13 cm), movable film barriers, and balance float were made of Teflon. The system was kept in a closed chamber with thermal regulation of ±0.1°C. Null balance sensitivity was ±0.04 dyn/cm and reproducibility was ±0.1 dyn/cm. Maximum uncertainty in the total surface area occupied by a monolayer was ±0.4%.

Procedures: Before each experiment, the apparatus was cleaned by rinsing with distilled water. Then fresh water was added to the trough and the surface swept. Solvent-spread films were formed on approximately 500 cm^2 of area by applying 25–50 μl solution directly to the water surface. At least 5 min was allowed for solvent evaporation. Solvent-spread films were aged from 5 min to 12 h before obtaining π-A data. Aging had no effects. To prepare solvent-free films, crystalline DML or DPL was placed in a cup-shaped container made of platinum wire gauze. The platinum cup was then lowered to the water surface and left in contact until a significant pressure was measured. The cup was then removed, and the surface area was expanded to about 500 cm^2, where π was zero. When formed from the crystals, the monolayer was not permitted to reach its equilibrium spreading pressure, to avoid possible collapse or

incorporation of excess crystals in the system. At least 5 min was allowed for DML films to stabilize when an isotherm above T_c was being sought. When films were cooled below T_c, the surface area remained at about 500 cm^2, $\pi = 0$, for 2–18 h before the isotherm was determined. The volume of water in the trough was kept constant so that π was unaffected by evaporation of water even after 18 h. π-A isotherms were reproducible within the limitations imposed by π-relaxation and manual operation of the apparatus; reproducibility for π was ± 0.1 dyn/cm.

Compression isotherms were obtained by manually advancing the film barrier at about 0.2 cm/s, interrupting compression every 1–2 cm for about 1 min to measure each π and A. π was read immediately after stopping the barrier and again 30 s later. This protocol was adopted for convenience and because it permitted a rough estimate of π-relaxation rates and film stability. The initial π readings are reported. At least two isotherms per experiment were obtained. A fresh monolayer was formed for each isotherm, although films were often expanded and recompressed as a test of repeatibility and film loss.

Leakage of film material behind the balance float was checked by expanding the film surface to 500 cm^2, and then decreasing the surface area behind the float with another barrier. Leakage of material behind the film barrier was checked by reducing the surface area behind the barrier to 2.5% of maximum by means of a rear guard barrier. This causes material to spill over the sides of the trough and increases the value of π if film has leaked behind the main film barrier. Talc was also placed behind the film barrier as a leak detector. 2 of 20 experiments were discarded because of leakage.

Contamination of the chloroform used was checked by spreading 50 μl solvent at 500 cm^2 area and compressing to 6.5 cm^2. No changes in π were detected.

Molecular areas ($Å^2$/molecule) of solvent-spread monolayers were calculated directly. Films formed from the liquid crystal phase were referenced to the solvent-spread films at a single π, where the matching π's coincided to within 0.1 dyn/cm and were as low as possible.

RESULTS

DML isotherms are presented in Fig. 1. At 31°C, the isotherms are stable (surface pressures decreased by 5% or less during the 30-s interval between readings and the rate of change slowed markedly during this time), and are independent of the method of film formation. Moreover, the π-A isotherm was reversible—compression and expansion curves were identical.

In contrast, a DML film formed from crystals at 27°C and cooled to 7.4°C is substantially different from a film spread from solvent at 7.4°C. The isotherm of the solvent-spread film shows an inflection at about 11 dyn/cm and 60 $Å^2$/molecule, and has a plateau width of about 15 $Å^2$/molecule. The isotherm of the crystal-formed film has an inflection at about 14 dyn/cm and 50 $Å^2$/molecule and has a plateau width of about 5 $Å^2$/molecule. Isotherms of the two films below about 5 dyn/cm were indistinguishable.

The stability characteristics of the solvent-spread and crystal-formed DML films also differed below T_c. At every π the 30-s relaxation of the crystal-formed film was greater than the solvent-spread film. This was best observed at the inflection point and at higher pressures. At the inflection, the crystal-formed film relaxed by 12% while the solvent-spread film relaxed by 10% in 30 s with no obvious slowing of the rates; these rates were reproducible. In one case, where a crystal-formed film was observed

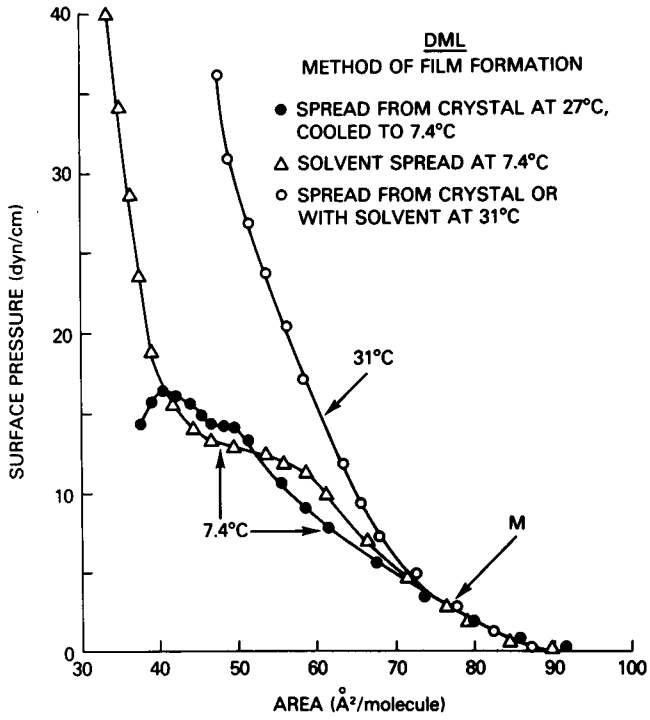

FIGURE 1 Surface pressure-surface area isotherms of (L-α) dimyristoyl lecithin monolayers. These are representative experiments. All data points are not included in plots. Films formed above T_c at 27°C from liquid crystals and cooled to 7.4°C are compared with films spread from solvent at 7.4°C. Films formed at 31°C from liquid crystal and solvent-spread films gave indistinguishable isotherms. M indicates reference point for calculation of molecular areas of crystal-formed film.

for 5 min at the inflection, π declined to 50% of its initial value. Further compression of the solvent-spread film up to 40 dyn/cm stabilized it. In contrast, the crystal-formed film could not be stabilized by further compression, and it actually collapsed at about 41 Å^2/molecule (Fig. 1).

Limitations of the apparatus prevented determination of DPL isotherms above T_c (41°C). However, DPL could be spread rapidly from the crystal phase at 42°C, and the resulting monolayer cooled. Solvent-spread and crystal-formed films were markedly different below T_c, as shown in Fig. 2. Inflections in the π-A isotherms of solvent-spread films occur at about 20 dyn/cm and 48 Å^2/molecule at 31°C, and at about 9 dyn/cm and 56 Å^2/molecule at 25.4°C. Plateau widths are about 6 and 10 Å^2/molecule, respectively. The crystal-formed film cooled to 25.4°C gave an isotherm with an inflection at about 22 dyn/cm and 39 Å^2/molecule, with a plateau width of about 2 Å^2/molecule. The two film types at 25.4°C were not identical even at very low π. The value of π for the crystal-formed film was consistently higher at all areas.

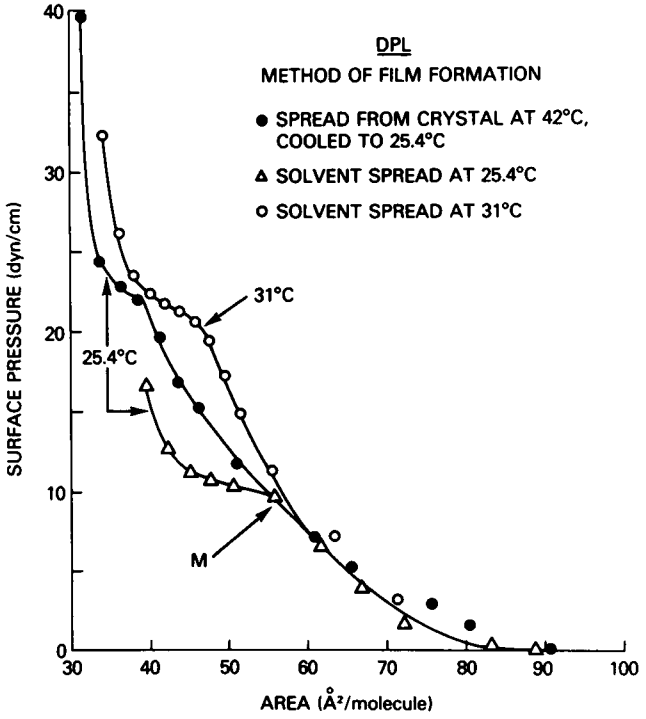

FIGURE 2 Surface pressure-surface area isotherms of (L-α) dipalmitoyl lecithin. Crystal-formed films were spread from liquid crystals above T_c at 42°C and cooled to 25.4°C. Solvent-spread films were formed at the isotherm temperatures. All data points are not included in plot of these representative experiments. M indicates reference point for calculation of molecular areas of crystal-formed films.

The instability traits observed for DML films were also seen for DPL. The rate at which π decreased was consistently higher for the crystal-spread than for the solvent-spread film under comparable conditions. The onset of the plateau region for each film was distinguished by more than a 10% decrease of π in 30 s with no obvious slowing of the rate of π relaxation. At the inflection π for the crystal-formed film relaxed by 18% and for the solvent-spread film by 13%. In one case a solvent-spread film at the inflection point was followed for 40 min; π declined to 14% of its initial value. Further compression stabilized only the solvent-spread film. However, at higher pressures, the rate of π relaxation slowed for the crystal-formed film, so that the surface layer could be compressed well beyond the plateau region before collapse if the barrier was stopped for less than 15 s for balance reading.

The π-A isotherms were not reversible. For both DML and DPL films below T_c, and at surface pressures that exceed π_e, the expansion isotherms did not retrace the compression isotherms. However, a compressed film could be re-expanded and compressed again to reproduce the first compression isotherm, provided the first compression did not proceed to surface collapse.

DISCUSSION

Monolayers of pure DML were formed by two independent methods, but otherwise treated identically. Compression π-A isotherms of the two film types for DML were compared. It was shown (Fig. 1) that above T_c (23.5°C) the isotherms were identical, that they could be obtained reversibly, and that the films were stable. π_e for DML at these temperatures (> 23.5°C) is approximately 49 dyn/cm (18). Since the maximum value of π for the 31°C isotherm was about 40 dyn/cm, π_e was never exceeded. Therefore, at 31°C, DML monolayers conform to criteria for equilibrium. It is evident that such films were not subject to artifacts caused by trace contaminants, solvent effects, or the presence of solid lecithin micro-crystals in the surface, which might cause measureable discrepancies in the π-A isotherms.

The equilibrium spreading pressure, π_e, for DPL and DML at temperatures below T_c is extremely small, about 0.1 dyn/cm (10, 18, 22), or less than the resolution of typical film balances. Since reported π values for most isotherms exceed π_e at temperatures below T_c, these films are in metastable states with respect to bulk lipid (17, 18, 22).

We have also demonstrated that films below T_c formed of the same material but by two independent methods have different properties, and therefore are not in the same state. For example discrepancies between π-A isotherms of crystal-spread and solvent-spread films below T_c are at least an order of magnitude greater than the sensitivity and reproducibility of the film balance. Indeed, the discrepancies are of the same order of magnitude as the alterations in the isotherm below T_c produced by a 5°C change in temperature (3, 11, 17, 20, 22). Moreover, different π-relaxation kinetics were observed for the two film types below T_c. The crystal-spread film was always more unstable than the solvent-spread film; the latter could be stabilized by compression beyond the inflection point, whereas the former could not. Since only the method of film formation was varied in these experiments, we conclude that metastable lecithin films below T_c are history-dependent. Apparently lecithin films below T_c can be trapped in a variety of metastable conditions by appropriate manipulation of their history.

Several opposing arguments may be raised against the foregoing discussion of our results:

Trace Impurities

It is thought that trace impurities can affect π-A isotherms below T_c profoundly (17, 23). We could not detect impurities in our lecithin samples by means of gas or thin-layer chromatography, and no criteria have been established for quantitative identification of trace impurity effects. Films above T_c showed no discrepancies. We used the same material to form the metastable films by independent pathways. The different pathways might have influenced the final impurity compositions of the metastable films, but the problem remains of choosing the "correct" isotherm. Of course, metastable systems are inherently sensitive to small disturbances or trace contaminants, so that it seems fruitless to pursue the issue unless trace contamination can

be identified equivocally. This requires measurement of the mole fractions of contaminants, which has not yet proved feasible. Sample cleanup procedures (17, 23) might be resorted to, but because trace quantities cannot be measured, the final sample might still be contaminated, perhaps by new substances introduced during the cleanup. Systematic use of a cleanup procedure seems to assure only that the final material has a well-defined history so that the corresponding π-A isotherms are reproducible.

Incomplete Spreading from Crystals and the Presence of Microcrystals

Lecithin microcrystals might have been trapped in the surface of our crystal-formed films, thus causing deviations from solvent-spread films. However, crystal-formed films were true equilibrium materials. They were expanded to $\pi = 0$ and kept in this state at least 5 min before cooling. Above T_c microcrystals cannot exist at $\pi = 0$. During cooling π was kept at 0 ($\pm$ 0.1 dyn/cm). Cooling time was from 2 to 18 h. Formation of microcrystals during cooling at $\pi = 0$ should be a random event, thus causing significant variability among isotherms of independently formed films. However, the isotherms we obtained were reproducible.

Equilibrium between Metastable Surface States

The fact that DML and DPL films are metastable at temperatures below T_c does not necessarily exclude the possibility that equilibria may exist among various surface film (albeit metastable) states. One might also argue that instability of π does not prevent measuring state functions if the relaxation times for externally imposed changes of the intensive variables are very short relative to the metastable decay. However, for this argument to be valid, the same material should exhibit a reproducible dynamic response to the same externally imposed changes of the intensive thermodynamic variables. Our observation that the π-relaxation kinetics are also history-dependent indicates that kinetic considerations are irrelevent, since these metastable states are not uniquely defined. This is to be expected of history-dependent, metastable materials.

There are considerable data in the literature consistent with our conclusion that the state of a lecithin surface film below T_c is arbitrary. We could compare isotherms below T_c reported by different laboratories, point by point, and show discrepancies inconsistent with the accuracy and reproducibility of the methods. However, there is always some uncertainty about true molecular areas, whereas surface pressure is measured directly and accurately. We shall focus upon the surface pressure measured at the onset of an apparent phase transition. This pressure, π_i, corresponds to a marked inflection point in the π-A isotherm and is identified easily. We note that π_i marks the onset only of an apparent phase transition, because there is no direct evidence for a true phase transition, even between two metastable states. According to the Gibbs phase rule, $d\pi/dA = 0$, whenever two coexistant phases are at equilibrium. But $d\pi/dA < 0$ for all areas, according to all data reported. Thus for all $\pi > \pi_i$ the isotherm must reflect strictly nonequilibrium properties. However, it could be argued that this is merely a consequence of the kinetics of the phase transition between two unique metastable states, so that yet other metastable

states, not in equilibrium, are produced (17) when the film is compressed beyond the area at π_i. But if we are to consider the transition as an equilibrium event, at least the parameter π_i should be known accurately, and should be unique for a given temperature.

Apparent phase transitions between liquid-expanded and condensed states of the surface film are seen only below T_c. The π_i of solvent-spread DPL films at 22°C depends on the solvent used, ranging between 5 and 9 dyn/cm (2). This range is about half the increment observed in π_i for a 5°C temperature change (11, 17, 20). The π_i of solvent-spread DPL at 25–26°C has been reported as about 7 dyn/cm (11, 20), 9 dyn/cm (22, this paper), 14 dyn/cm (17), or as nonexistent at surface areas larger than about 45 $Å^2$/molecule (8, 23). DML films show no transition at 22°C (3, 7, 17, 21) although the reported π's at any area differ by as much as a factor of 2. The π_i of solvent-spread DML at 5°C has been reported as about 6 dyn/cm (3) or as nonexistent (7), while we observed a π_i of about 11 dyn/cm at 7°C.

These discrepancies may seem inconsequential at first glance. However, we note that the data were generated by basically the same techniques, so that the films had similar histories. If films below T_c were in unique states, then discrepancies among π_i measurements would be of the same magnitude as the standard error of a single series of measurements in one laboratory. Isotherm reproducibility is usually at least $\pm$ 1 dyn/cm, and this is seen routinely for equilibrium films above T_c.

The seemingly small discrepancies at the isotherm inflection actually are very significant when the energetics of the apparent phase transitions are considered. An important reason for measuring isotherms, of course, is to estimate energies and entropies of the monolayer states to gain some ideas of the molecular structures of the films and the physical factors that control the structures. If two states are in equilibrium, a form of the Clausius-Clapeyron equation can be used to estimate the latent heat of the phase transition, and thereby the entropy of the transition. This approach has been used with great success when evaluating equilibrium transitions between surface gaseous and liquid-expanded states, although its validity for analysis of metastable state transitions has been questioned (9). We need to estimate $d\pi_i/dT$ to calculate apparent latent heats of transition. Approximations of this quantity can be calculated for solvent-spread DPL films based on π_i estimates between about 25° and 35°C. Our own data yield a value of about 2 dyn/cm-°C, as do the data of Villalonga (22), while other data yield values of 1.8 (11), 1.6 (17), and 1.4 (20). We believe this range of values is broader than can be tolerated for meaningful thermodynamic analysis. If an area difference between the expanded and condensed states at 25°C were approximately 15 $Å^2$/molecule DPL (11, 17, 22), the latent heat range associated with this range of $d\pi_i/dT$ is about 9–13 kcal/mol. Such a large energy range is consistent with the existence of many metastable states below T_c.

The lack of unique metastable states of lecithin surface films below T_c poses serious difficulties for specific molecular explanations of film properties by means of equilibrium statistical mechanics, such as proposed by Marcelja (14), Nagle (15), and Scott (19). The surface layer might be a useful model for more complicated biological sys-

tems if correspondence of the model and the natural system could be established. This is a difficult task, given the degeneracy of metastable states of the model below T_c. The utility of π-A isotherms of surface films for studies of apparent phase transitions and of effects of other lipids such as cholesterol on them for elucidation of cell membrane properties is thus questionable. Similarly, a DPL monolayer below 41°C may not be a reliable model for the interfacial film present in a pulmonary alveolus. Certainly explanation of lung volume-inflation pressure hysteresis in terms of apparent phase transitions observed for DPL films at 20°C (20) does not seem warranted.

Cell membranes and alveolar surfactant are not composed of a single pure lecithin but are complex mixtures of materials. Dissection of the mixture and study of the surface properties of its components could lead to misleading conclusions unless great care is taken first to define the interfacial properties of mixtures containing lecithins. Due considerations for thermodynamic equilibrium and the uniqueness of metastable states is essential.

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REFERENCES

1. BIENKOWSKI, R., and M. SKOLNICK. 1972. Dynamic behavior of surfactant films. *J. Colloid Interface Sci.* **39**:323–330.
2. CADENHEAD, D. A., and B. M. J. KELLNER. 1974. Some observations on monolayer spreading solvents with special reference to phospholipid monolayers. *J. Colloid Interface Sci.* **49**:143–145.
3. CADENHEAD, D. A., and M. C. PHILLIPS. 1968. Molecular interactions in mixed monolayers. *Adv. Chem. Ser.* **84**:131–148.
4. CHAPMAN. D. 1975. Phase transitions and fluidity characteristics of lipids and cell membranes. *Q. Rev. Biophys.* **8**:185–235.
5. CHAPMAN, D., R. M. WILLIAMS, and B. D. LADBROOKE. 1967. Physical studies of phospholipids. VI. Thermotropic and lyotropic mesomorphism of some 1,2-diacylphosphatidyl cholines (lecithins). *Chem. Phys. Lipids.* **1**:445–475.
6. CLEMENTS, J. A. 1962. Surface phenomena in relation to pulmonary function. *Physiologist.* **5**:11–28.
7. DEMEL, R. A., L. L. M. VAN DEENEN, and B. A. PETHICA. 1967. Monolayer interactions of phospholipids and cholesterol. *Biochim. Biophys. Acta.* **135**:11–19.
8. GALDSTON, M., and D. O. SHAH. 1967. Surface properties and hysteresis of dipalmitoyl lecithin in relation to the alveolar lining layer. *Biochim. Biophys. Acta.* **137**:255–263.
9. GERSHFELD, N. L. 1976. Physical chemistry of lipid films at fluid interfaces. Annu. Rev. Phys. Chem. **17**:349–368.
10. GERSHFELD, N. L., and K. TAJIMA. 1977. Energetics of the transition between lecithin monolayers and bilayers. *J. Colloid Interface Sci.* In press.
11. HUI, S. W., M. COWDEN, D. PAPAHODJOPOULOS, and D. F. PARSONS. 1975. Electron diffraction study of hydrated phospholipid single bilayers. Effects of temperature, hydration and surface pressure of the "precursor" monolayer. *Biochim. Biophys. Acta.* **382**:265–275.
12. KING, R. J., and J. A. CLEMENTS. 1972. Surface active materials from dog lung. II. Composition and physiological correlations. *Am. J. Physiol.* **223**:715–726.
13. KING, R. J., and J. A. CLEMENTS. 1972. Surface active materials from dog lung. III. Thermal analysis. *Am. J. Physiol.* **223**:727–733.
14. MARCELJA, S. 1974. Chain ordering in liquid crystals. II. Structures of bilayer membranes. *Biochim. Biophys. Acta.* **367**:165.
15. NAGLE, J. F. 1976. Theory of lipid monolayer and bilayer phase transitions. Effect of head group interactions. *J. Membr. Biol.* **27**:233–250.

16. PAGANO, R. E., R. J. CHERRY, and D. CHAPMAN. 1973. Phase transitions and heterogeneity in lipid bilayers. *Science* (Wash. D.C.). **181**:557–559.
17. PHILIPS, M. C., and D. CHAPMAN. 1968. Monolayer characteristics of saturated 1,2-diacylphosphatidylcholine (lecithins) and phosphatidylethanolamines at the air-water interface. *Biochim. Biophys. Acta.* **163**:301–313.
18. PHILIPS, M. C., and E. A. HAUSER. 1974. Spreading of solid glycerides and phospholipids at the air-water interface. *J. Colloid Interface Sci.* **49**:31–39.
19. SCOTT, H. L., JR. 1975. A theoretical model for lipid monolayer phase transitions. *Biochim. Biophys. Acta.* **406**:329.
20. TRÄUBLE, H., H. EIBL, and H. SAWADA. 1974. Respiration—a critical phenomenon? Lipid phase transitions in the lung alveolar surfactant. *Naturwissenschaften.* **61**:344–354.
21. VAN DEENEN, L. L. M., U. M. T. HOUTSMILLER, G. H. DEHAAS, and E. MULDER. 1962. Monomolecular layers of synthetic phosphatides. *J. Pharm. Pharmacol.* **14**:429–444.
22. VILLALONGA, F. 1968. Surface chemistry of L-α-dipalmitoyl lecithin at the air-water interface. *Biochim. Biophys. Acta.* **163**:290–300.
23. WATKINS, J. C. 1968. The surface properties of pure phospholipids in relation to those of lung extracts. *Biochim. Biophys. Acta.* **152**:293–306.